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Separation and purification of chlorogenic acid by molecularly imprinted polymer monolithic stationary phase

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Abstract

Separation and purification of chlorogenic acid by removal of the impurities compounds co-existed in the product using molecular imprinting technique was firstly reported. In this work, an in situ synthesis method was utilized for the preparation of molecularly imprinted polymer monolithic stationary phase using the impurity molecule (caffeic acid) as template, the mixture of tetrahydrofuran and isooctane as solvent, and methacrylic acid and ethyl glycol dimethacrylate as functional monomer and cross-linker, respectively. The retention behavior of the monolithic polymer to chlorogenic acid molecule, the template and several main impurities compounds in the product was studied and the adsorption capacity of compounds on the stationary phase determined by frontal chromatographic technique. A relatively weak retention of the target product molecule (chlorogenic acid) on the polymer and a strong adsorption capability of the monolith to the template and several main impurities were observed. This might mainly result from the 'shape' difference of chlorogenic acid molecule with the impurities compounds molecules. This approach was shown to be successful for the separation and purification of chlorogenic acid from the extract of *Eucommia ulmodies* leaves.

Keywords: Molecularly imprinted polymer monolith; In situ synthesis; Chlorogenic acid; Caffeic acid; Separation

1. Introduction

Molecularly imprinting is a technique used for preparing polymer by arranging functional monomers around a template compound and by fixing it with cross-linker in solution. Removal of the template from the obtained polymer matrix produces vacant recognition sites that exhibit recognition ability and have a predesigned selectivity to the template molecule and structurally related compounds [1,2]. Lately, molecularly imprinted polymers (MIP), as chromatographic separation media for pharmaceutical analysis and environmental detection [3–5], and even as a new material in solid-phase extraction (SPE) for enriching bioactive compounds and chiral medicinal molecule of interest by the selectively re-binding of the polymer to analytes [6–8], have attracted growing attention. Especially, its capability of molecular recognition, easiness to prepare, and tolerance to harsh environmental conditions, like high temperature, high pressure, acid, base, and even organic solvent, makes MIP a very promising separation material in SPE and sample preconcentration. In the pre-concentration of bupivacaine from plasma samples, MIP showed a higher affinity than C_{18} material [9]. For the widespread application of the MIP, however, a potential drawback is the need for large amount of pure template in the preparation of it [10]. In situ synthesis technique, developed by Svec and Frechet [11] and used by Matsui to prepare MIP in chromatographic column [12], is a good solution for this problem, which not only greatly lowered the consumption of the template but also was a convenient and single-step production procedure enabling us to obtain a column packed with MIP without any tedious step [13]. Simultaneously, the MIPs monolith obtained by this technique was also shown to have high selectivity and good flow-through properties [14–16]. No doubt, application of this kind of molecularly imprinted polymer column for the enrichment and purification of small molecules is a beneficial attempt.

Chlorogenic acid, an important bioactive compounds and rich in the leaves of *Eucommia ulmodies* (a precious Chinese materia medica), has anti-bacterial, phlogistic, mutagenic, oxidant

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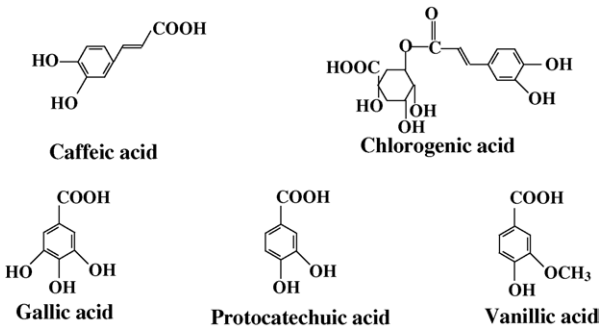


Fig. 1. The molecular structure of the investigated substances.

and other biological activities [17]. A further pharmacological study needs high purity of chlorogenic acid [18]. Extraction of chlorogenic acid from the leaves of this plant is an efficacious approach to obtain this compound [19]. However, in the product obtained by this approach, there also exist some compounds with similar chemical activity to chlorogenic acid, like caffeic acid, gallic acid, protocatechuic acid, vanillic acid, and so on [20]. Due to the existence of similar functional groups in their molecules as shown in Fig. 1, these compounds belong to penylpropanoid compounds [21]. This makes it rather difficult to purify chlorogenic acid from the *E. ulmodies* leaves extract. Even so, some researches make arduous efforts to get chlorogenic acid of high purity. The conventionally used chemical separation technique was time- and solvent-consuming and was a complicated process [22,23]. Thin layer chromatography method can only obtain a tiny amount of target product and the preparative liquid chromatography separation technique is a good but expensive approach to purify chlorogenic acid with the need of expensive stationary phase material [24,25]. Development of a new technique for large-scale separation and purification of chlorogenic acid is thus of essential importance for the potential application of chlorogenic acid.

Based on the functional groups and the molecular geometric shape of chlorogenic acid (target product) and co-existed compounds mentioned as above (regarded as the impurities) in the *E. ulmodies* leaves extract [26], it is suitable to design specific recognition cavities with no-covalent approach. Thus, we directed our attention to applying molecular imprinting technique. Furthermore, a crucial question needs to be taken into account is the choice of the template compound for the preparation of the molecularly imprinted polymer. After study on the chemical constitution of the *E. ulmodies* leaves extract and the molecular structure character of chlorogenic acid and the impurities compounds, it was found that there exist a great 'shape' difference between chlorogenic acid molecule and the impurities molecules and a relatively small 'shape' difference among the impurities molecules [27–29]. This offered us a new idea to utilize the impurity molecule as the template for the preparation of the MIP, and such MIP was then applied for the purification of chlorogenic acid by the weak retention of the product molecule on the MIP. Thus, caffeic acid molecule, a typical compound in the impurities, was chosen as the template for the preparation of the MIP. Such work was not yet reported to date in the literature.

In present work, the MIP monolithic stationary phase was prepared in chromatographic column by an in situ synthesis method using caffeic acid as the template. The retention behavior of chlorogenic acid, the template and the other impurities molecules co-existed in the *E. ulmodies* leaves extract on the MIP monolith was studied. The adsorption capacity of the monolithic polymer to these compounds was determined by frontal analysis technique. The monolith was successfully applied to the separation and purification of chlorogenic acid from the leaves extract of *E. ulmodies*, resulting in high purity chlorogenic acid.

2. Experimental

2.1. Chemicals and materials

Caffeic acid (CA), gallic acid (GA), protocatechuic acid (PCA), vanillic acid (VA), chlorogenic acid (CGA), methacrylic acid (MAA) and ethyl glycol dimethacrylate (EDMA) were purchased from Sigma company. 2,2′-Azobisisobutyronitrile (AIBN), tetrahydrofuran (THF), isooctane and acetone were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Glacial acetic acid (HAc), lead acetate ($Pb(OAc)_2$), H_2SO_4, acetonitrile (MeCN) and methanol (MeOH) was from Hunan Normal University Reagent Factory (Hunan, China). All the reagents are analytical-grade. The monomers before use have been purified by distillation. Stainless steel column (200 mm × 4.6 mm I.D. geometrical volume 3.32 mL) was offered by Beijing Jinouya Science & Development Co. (Beijing, China).

E. ulmodies leaves extract was prepared by the modified method as ref. [30]. 50.0 g of *E. ulmodies* leaves dried in air was crushed and refluxed in $CHCL_3$ for 2 h. The filter residue was extracted with methanol for one hour and then filtrated. The filter liquor was processed with $Pb(OAc)_2$ and then filtrated. The yellow solid obtained was treated with 5% H_2SO_4 (pH 2). After removal of the solid substrates, the liquid was concentrated in vacuum and then extracted with ethyl acetate for three times. After recovery of ethyl acetate, the product obtained was dried in vacuum and restored in the refrigerator. HPLC analysis showed that the contents of chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid and vanillic acid in the extract were 85.6, 4.5, 1.3, 1.4 and 2.1%, respectively.

2.2. Preparation of caffeic acid imprinted polymers monolithic stationary phase

Caffeic acid (0.2 mmol) as the template, MAA (0.8 mmol) as the functional monomer, EDMA (2 mmol) as the cross-linker and AIBN (0.08 mmol) as the initiator were dissolved in a mixted solvent of THF and isooctane (1:2, v/v). The solution was deoxygenated with a stream of nitrogen gas for 20 min and then poured into a stainless column. In situ polymerization was initiated by heating the column in a water bath at 60 °C for 12 h. Afterwards, the template was washed with MeOH-HAc (9:1, v/v), MeOH and MeCN, sequently, at a eluent rate of 0.2 mL/min, until a stable baseline was obtained. As a control, a blank polymer monolith

without the template molecule was prepared and treated in the same manner.

2.3. Apparatus

A high-performance liquid chromatography (HPLC) system modified from HIC-6A ion chromatographer was used (Shimadzu, Japan). The system is equipped with a LP-6A solvent delivery pump, a SIL-6B auto-injector, a SCL-6B system controller, a column oven, a LC-10 UV model UV detector and a chromatographic workstation with a data-collected card (Dalian Chromatographic Instrument Co., China).

2.4. Chromatographic evaluation of the monolith

To evaluate the selectivity of the monolith, the retention behavior of analytes on the MIP monolithic stationary phase was studied at room temperature. After the column was equilibrated with mobile phase, 10 μL sample solution containing 0.04 mg/mL of analyte in MeOH was injected into the chromatographic column and eluted with acetonitrile as mobile phase at a rate of 0.2 mL/min, recording the retention time of analyte (detection wavelength: 280 nm). Each analyte was determined repeatedly for three times to assure the chromatographic reproducibility. Acetone was used for the measurement of void time (t_0). The retention factor (k) was calculated as $(t_R - t_0)/t_0$, where t_R is the retention time of the analyte. The selectivity factor (α) was defined as the ratio of the retention factor of the template molecule to the analogues ($\alpha = k_{template/analogue}$).

The adsorption capacity of the monolith to compounds tested was studied by frontal liquid chromatography, eluting the imprinted column with acetonitrile solution with analyte concentration ranging from 0.005 to 0.7 mg/mL. First, the column was equilibrated with acetonitrile as mobile phase. Then, the acetonitrile solution containing the lowest analyte concentration was pumped into the column at a rate of 0.2 mL min^{-1} and frontal development took place until a stable plateau was reached, i.e. completion of a breakthrough. After the column was re-equilibrated with acetonitrile, another acetonitrile solution containing the next higher analyte concentration was pumped into the column as above. By this way, a series of breakthrough curves were obtained for each analyte. The breakthrough volume was obtained from the half height method [31].

2.5. Separation procedure of chlorogenic acid by MIP monolith

The separation was carried out using an on-line style chromatography system. Prior to each injection, the MIP monolith column was equilibrated with mobile phase. Then, 50 μL of model sample solution containing chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid and vanillic acid (5.0 mg/mL for each substrate) was injected into the chromatography system and the column was eluted with mobile phase. The chlorogenic acid eluate was collected followed by evaporation to dryness using rotary evaporator for purity analysis.

Real sample solution before injection was prepared by dissolving 10 mg *E. ulmodies* leave extract in 1 mL methanol. The separation of chlorogenic acid was processed by the method mentioned as above.

2.6. HPLC analysis

HPLC analysis was carried out on C_{18} column. Mobile phase used was a mixture of methanol:water:acetic acid (20.3:78.7:1.0, v/v) at a rate of 1.0 ml/min. Injection volume was 10 μL and UV detection was at 280 nm. Each analysis was repeated for three times. Standard curve method was used for quantification.

3. Results and discussion

3.1. Preparation of the molecularly imprinted polymer monolithic stationary phase

In spite of the simplicity of the preparation process of molecularly imprinted polymer monolithic stationary phase, a number of factors have to be taken into account. Among these factors, the choice of the porogenic solvent is of significance for the preparation of the monolith. Huang et al. [13] studied the influence of porogenic solvent on the performance of the molecularly imprinted monolithic stationary phase. In our study, the mixture of tetrahydrofuran and isooctane is used as solvent for the preparation of the monolith. Furthermore, the ratio of isooctane to tetrahydrofuran in solvent needs to be optimized to get enough penetrability of the monolithic stationary phase. By testing the backpressure of the monolithic stationary phase prepared with different ratio of isooctane to tetrahydrofuran as a function of flow rate of acetonitrile, it can be found that the molecularly imprinted polymer monolith prepared with 3:1 (v/v) of isooctane to tetrahydrofuran has the lowest backpressure at the same flow rate of acetonitrile shown as the left inset in Fig. 2. Higher ratio

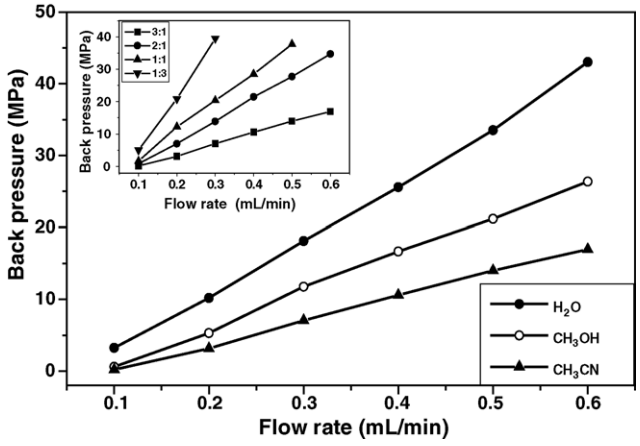


Fig. 2. Back pressure as a function of mobile phase flow velocity in the molecularly imprinted polymers monolith column with water (solid circles), methanol (open circles) and acetonitrile (up triangles) as mobile phase, respectively (main Figure). In the left inset: back pressure on the molecularly imprinted polymer monolithic stationary phase prepared with 3:1, 2:1, 1:1 and 1:3 (v/v) of isooctane to tetrahydrofuran as a function of acetonitrile flow rate.

of isooctane than 75% in the isooctane–tetrahydrofuran mixture solvent decreases the solubility of the template remarkably. Hence, 3:1 (v/v) of isooctane to tetrahydrofuran was selected as solvent for the preparation of the molecularly imprinted polymer monolithic stationary phase. Main Figure in Fig. 2 also gives the backpressure of the molecularly imprinted polymer monolithic stationary phase prepared under the optimized conditions using acetonitrile, methanol and water as mobile phase, respectively. Acetonitrile produced the lowest backpressure at the same flow rate of mobile phase and was chosen as mobile phase in the following experiments. In addition, a flow rate of mobile phase of 0.2 ml/min was chosen in our experiments, it resulted in a low column pressure and high separation factor.

3.2. The selectivity of the molecularly imprinted polymer monolith stationary phase

Aimed at application, the selectivity of the molecularly imprinted polymer monolithic stationary phase was studied by liquid chromatography, by eluting chlorogenic acid, the template molecule and several main impurities compounds co-existed in *E. ulmodies* extract with acetonitrile as mobile phase. The k and α values are given in Table 1. It is observed that the MIP monolithic stationary phase has the weakest re-binding ability to chlorogenic acid molecule and a strong adsorption ability to the template and structurally related compounds. This result may be explained by the nature of the molecularly imprinted polymer monolith and molecular intrinsic affinity. The strong retention of the polymer monolith to the template (caffeic acid) molecule results from the complementary binding sites in polymer matrix to the shape, size and functional group of caffeic acid molecule. For the other impurities compounds, the difference of position and number of functional groups in the molecule may also leads to different retention behavior on the polymer [32]. Furthermore, the weak compatibility of binding sites in monolith with the chlorogenic acid molecule in shape, size and functional group, especially in shape, leads to the first elution of chlorogenic acid out of the column. In addition, the lower retention and selectivity factors of the analytes on the control monolith than on the MIP monolith shows that there exist specified binding sites on the MIP monolith which take on strong adsorption ability to the tested compounds. This offered us a possibility of separation and purification of chlorogenic acid by removal of the main impurities (caffeic acid, gallic acid, protocatechuic acid and vanillic acid) co-existed in the *E. ulmodies* leaves extract with the caffeic acid imprinted polymer monolithic stationary phase.

Table 1
Retention factors (k) and selectivity factors (α) of the MIP and control monolith to substrates from liquid chromatography[a]

Substrates	k (MIP)	k (control monolith)	α (MIP)	α (control monolith)
Caffeic acid	9.48	1.27	1	1
Gallic acid	10.02	1.54	0.95	0.82
Protocatechuic acid	5.11	0.98	1.86	1.29
Vanillic acid	3.27	0.80	2.90	1.58
Chlorogenic acid	2.06	0.51	4.60	2.49

[a] Chromatographic conditions: mobile phase, CH_3CN; flow rate, 0.2 mL/min.

Table 2
The influence of acetic acid in acetonitrile on the retention behavior of substrates on the caffeic acid imprinted polymers monolith[a]

Acetic acid content (v/v) (%)	Parameter	CA	GA	PCA	VA	CGA
0	k	9.48	10.02	5.11	3.27	2.06
	α	1	0.95	1.86	2.90	4.60
2	k	3.38	3.41	1.58	1.04	0.66
	α	1	0.99	2.13	3.24	5.14
5	k	1.85	1.86	1.38	0.89	0.57
	α	1	0.99	1.34	2.08	3.25
7	k	1.34	1.55	1.12	0.68	0.42
	α	1	0.86	1.20	1.98	3.16
10	k	0.90	0.92	0.90	0.50	0.31
	α	1	0.98	1.00	1.81	2.91

[a] CA, caffeic acid; GA, gallic acid; PCA, protocatechuic acid; VA, vanillic acid; CGA, chlorogenic acid.

To improve the elution efficiency of substrates on the stationary phase, a little amount of acetic acid is added into the acetonitrile mobile phase. The influence of different amounts of acetic acid in acetonitrile on retention and selectivity factor is given in Table 2. It is seen that when the content of acetic acid in acetonitrile is increased from 0 to 10%, the selectivity factors firstly increase and then decrease, with a maximum value at 2% acetic acid, the retention factors decrease for each analyte. The competitive adsorption of the acetic acid molecule with the tested compounds on the binding sites is responsible for this result. Usually, the recognition of binding sites to the test compounds based on the hydrogen bond interaction is decreased by the addition of proton donor solvent. The result in Table 2 reflects this competitive adsorption of acetic acid molecules on the monolith with the increased acetic acid concentration in eluent, resulting in the decrease of the selectivity factor of tested compounds. However, acetic acid in the mobile phase can also accelerate the desorption of adsorbed analyte from the binding sites during elution, resulted in the decrease of retention time of analyte on the column and in the elimination of peak broadening and tailing. The mobile phase containing 2% acetic acid gave better selectivity factor and produces better resolution. Therefore, 2% acetic acidic acetonitrile solution was used as the mobile phase.

Fig. 3a shows the workability of the caffeic acid imprinted polymer monolithic stationary phase to separate the model mixture of chlorogenic acid and caffeic acid using 2% acetic acidic in acetonitrile solution as eluent and Fig. 3b shows that of the control monolith. Apparently, the molecularly imprinted monolithic stationary phase can completely separate the chlorogenic acid and caffeic acid molecule from their mixture. However, for the control monolith, only a single peak is observed on chromatogram, showing no separation ability to these two compounds.

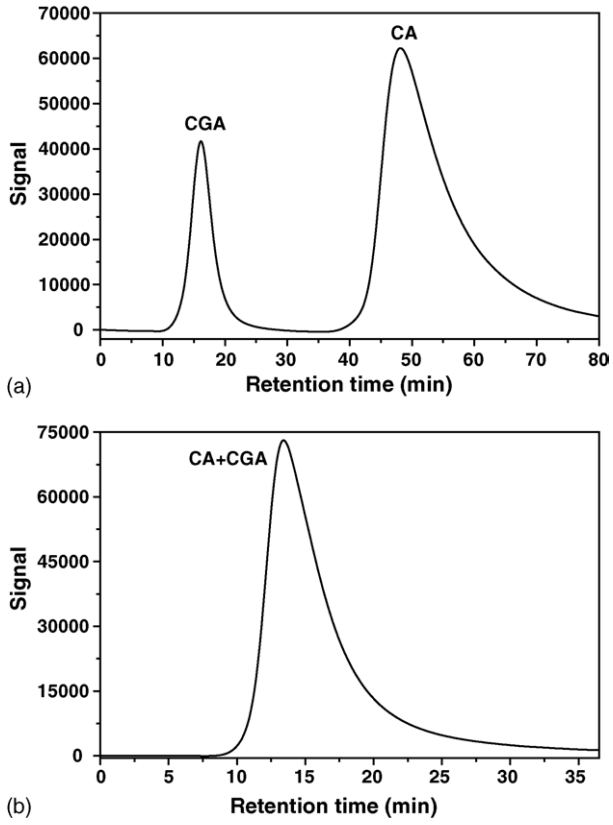


Fig. 3. Chromatograms for separation of caffeic acid (CA) and chlorogenic acid (CGA) on the caffeic acid imprinted polymer monolith stationary phase (a) and on the control monolith (b). *Conditions*: 2% acetic acid in acetonitrile as mobile phase; flow rate, 0.2 mL/min; injection volume, 10 μL; sample concentrations, 0.4 mg/mL in methanol.

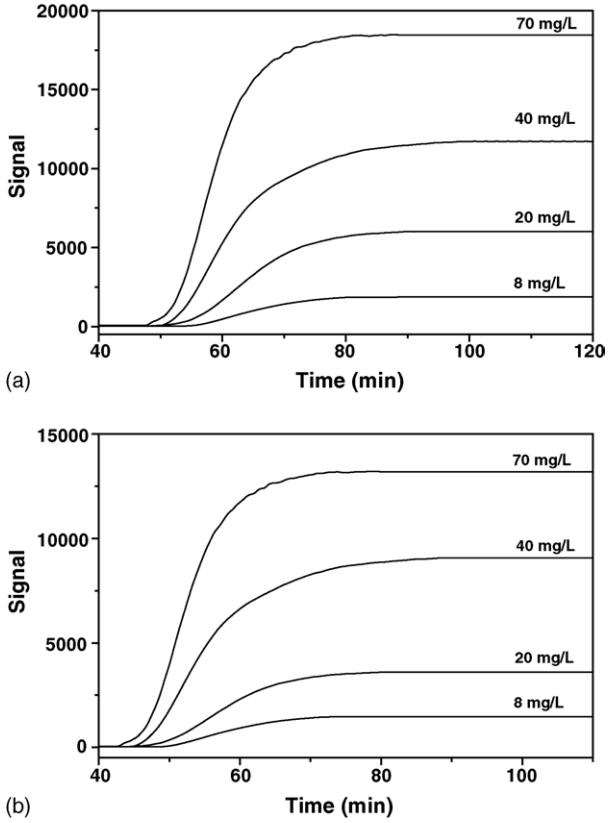


Fig. 4. The experimental breakthrough curves of caffeic acid (a) and chlorogenic acid (b) on the molecularly imprinted polymer monolithic stationary phase at different substrate concentrations in 2% solution of acetic acidic in acetonitrile with a flow rate of 0.2 mL/min.

3.3. Binding capacity of the molecularly imprinted polymer monolithic stationary phase

Study on the binding capacity of the molecularly imprinted polymer monolithic stationary phase is very helpful to verify the workability of the MIP monolith to separate chlorogenic acid from the extract. In this study, the experimental breakthrough curve of chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid and vanillic acid on the MIP monolith were measured by frontal chromatography in which the affinity constant of the polymer to substrates, the amount of binding sites and sites distribution on the surface of the polymer contributed to the position of the breakthrough point on the time axis in the chromatogram. As a typical example, some of the experimental breakthrough points of caffeic acid and chlorogenic acid on the imprinted polymer were obtained by the half height method from corresponding experimental breakthrough curves shown in Fig. 4. These points correspond to the amount of substrates retained on the monolith. By comparing the positions of the breakthrough point of caffeic acid (Fig. 4a) with that of chlorogenic acid (Fig. 4b) on the molecularly imprinted polymer monolith, it can be found that the rebinding ability of chlorogenic acid on the monolith is apparently weaker than that of caffeic acid, for example, at the same substrate concentration of 8 mg/L, a 1.2 mL lower breakthrough volume of chlorogenic acid than caffeic acid is observed.

From an adequate number of experimental breakthrough points within a well-defined range of substrate concentrations, the adsorption isotherms of five compounds tested on the molecularly imprinted polymer monolith are obtained as shown in the main Figure in Fig. 5. It shows, just as we expect, that within the concentration range studied, the monolith has the weakest adsorption capacity to the chlorogenic acid molecule, and takes on stronger retention to caffeic acid and the other structurally related compounds at the same substrate concentration. Fig. 5 also gives the best fitting of the experimental data of the caffeic acid (left inset in Fig. 5) and chlorogenic acid (right inset in Fig. 5) to Langmuir equation, Bi-Langmuir equation and Freundlich equation [33], respectively. For these two compounds, the isotherm obtained by fitting the data to Langmuir model is of a quality inferior to the Freundlich model and the Bi-Langmuir model. The fitting of the data to the Freundlich equation is slightly better than to the Bi-Langmuir equation. From the best coefficients of the Bi-Langmuir fit, it can be gained that the concentrations of the selectively binding sites on the molecularly imprinted polymer monolithic stationary phase are 0.22, 0.16, 0.09, 0.08 and 0.02 mmol/L for caffeic acid, gallic acid, protocatechuic acid, vanillic acid and chlorogenic acid, respectively. This indicates the feasibility to separate and purify chlorogenic acid from their mixture within a certain range of sample amount, by the selectively rebinding of the molecularly imprinted polymer monolithic stationary phase to the substrates.

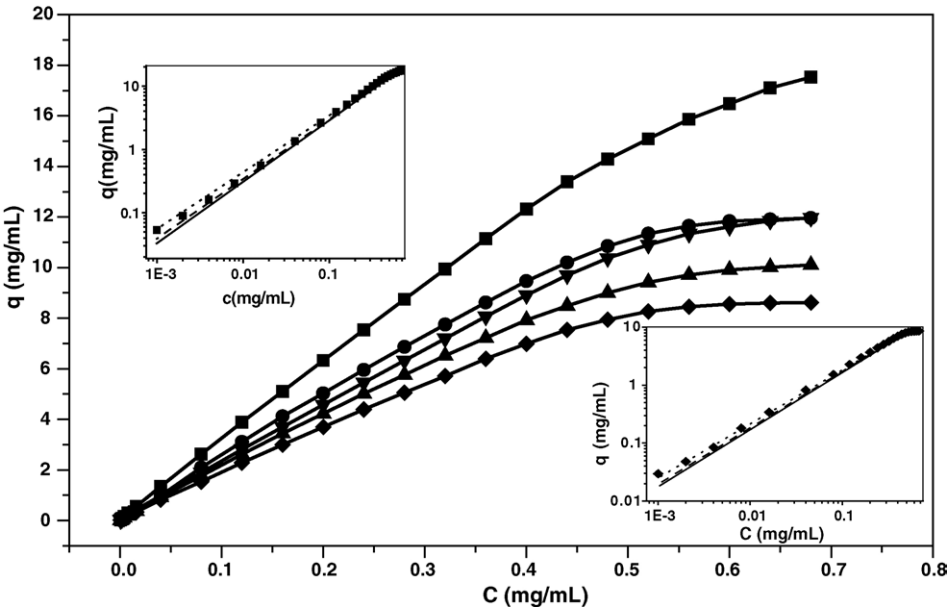


Fig. 5. Experimental isotherms (main Figure) of caffeic acid (solid squares), gallic acid (solid circles), protocatechuic acid (down triangles), vanillic acid (up triangles) and chlorogenic acid (diamonds) on the imprinted polymers monolith and the fittings of experimental data of caffeic acid (left inset) and chlorogenic acid (right inset) to Langmuir equation (solid lines), Bi-Langmuir equation (dash lines) and Freundlich equation (dash dot lines).

3.4. On-line separation of chlorogenic acid

Due to the weak retention of chlorogenic acid molecule on the molecularly imprinted polymer monolithic stationary phase, the chlorogenic acid was firstly eluted out of the polymer column and separated from the other constituents using acetonitrile containing 2% acetic acid as eluent. However, from the analysis as above, the sample volume for each injection needs to be optimized in order to obtain the fraction with high content of chlorogenic acid. Fifty microliters of model sample solution containing chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid and vanillic acid (5.0 mg/mL for each substrate) was injected into the polymer column, the elution chromatogram obtained is given in Fig. 6. It can be seen that the chlorogenic acid is eluted out of the column within the time range between 10

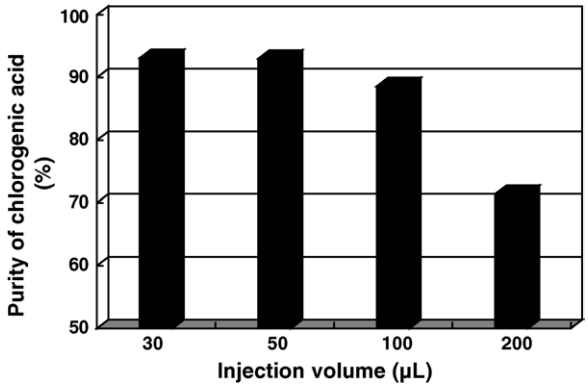


Fig. 7. The influence of injection volume on the purity of chlorogenic acid in dried fraction.

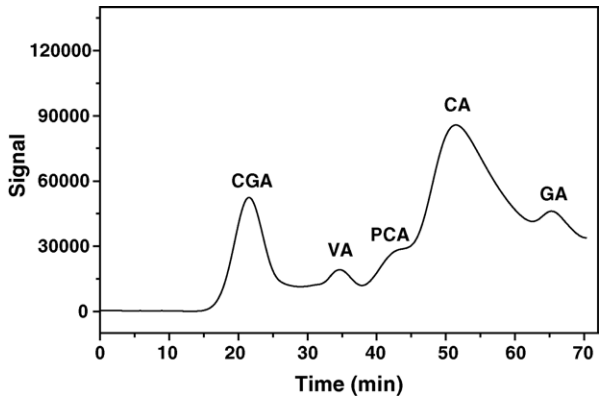


Fig. 6. The elution chromatogram of the model mixture of chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid and vanillic acid on the molecularly imprinted polymer monolithic stationary phase column using 2% acetic acidic acetonitrile as eluent at a rate of 0.2 mL/min.

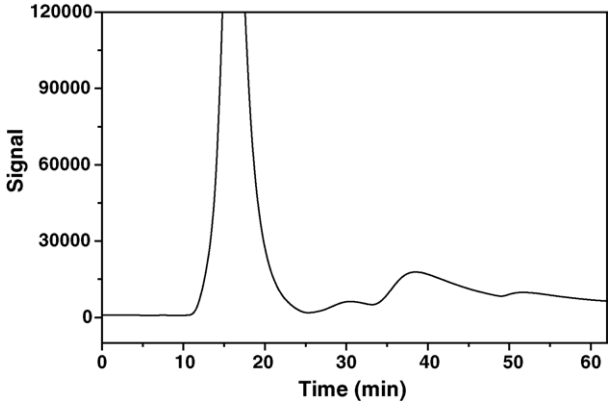
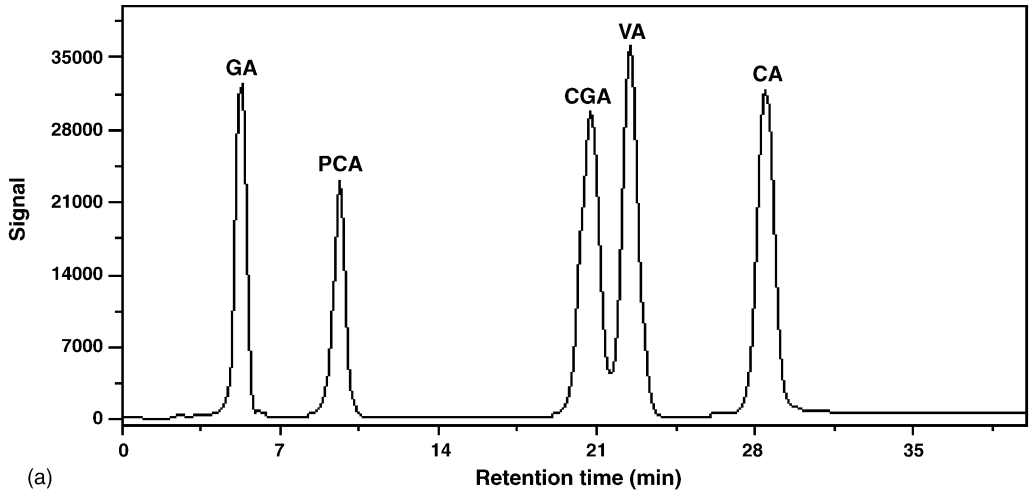
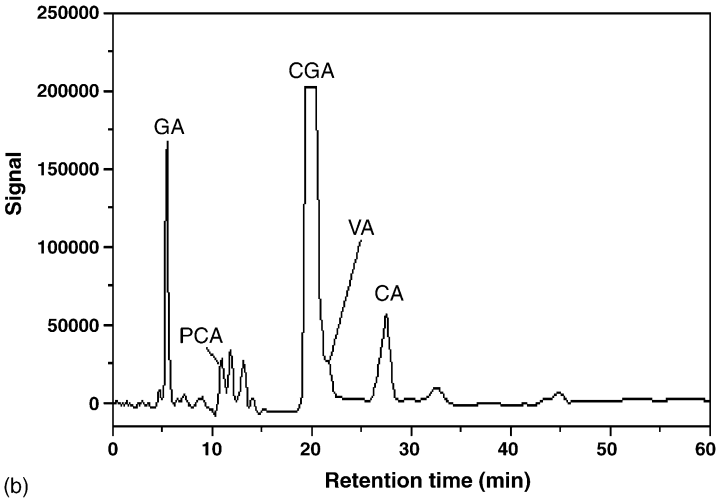


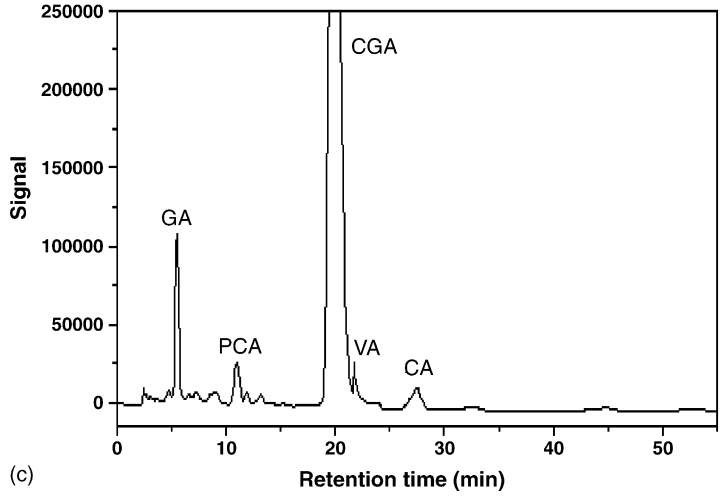
Fig. 8. The elution chromatogram of *Eucommia ulmodies* leaves extract on the molecularly imprinted polymer monolith stationary phase. *Conditions*: 2% acetic acid acetonitrile as mobile phase; flow rate, 0.2 mL/min; injection volume, 50 μL.





Peak Integration Table

Retention time (min)	Peak area	Content (%)
2.26	900	0.05
4.25	360	0.02
5.60	26111	1.45
7.32	4862	0.27
8.57	5582	0.31
9.01	4152	0.23
10.3	23049	1.28
11.2	10264	0.57
12.6	14586	0.81
13.1	3962	0.22
20.3	1545025	85.8
21.0	36555	2.03
28.9	82293	4.57
32.5	19808	1.10
44.8	23229	1.29
Total	1800729	100.00



Peak Integration Table

Retention time (min)	Peak area	Content (%)
2.71	789	0.03
2.95	1051	0.04
3.01	3152	0.12
5.62	8668	0.33
7.55	2364	0.09
8.09	1839	0.07
9.25	4728	0.18
10.2	12082	0.46
10.9	6829	0.26
13.2	3940	0.15
13.7	2889	0.11
19.8	2534592	96.5
20.9	23113	0.88
28.4	14446	0.55
32.7	2364	0.09
45.2	3677	0.14
Total	2626520	100.00

Fig. 9. Chromatograms of standards (a), *Eucommia ulmodies* leaves extract (b) and the fraction (c) on C_{18} column. *Conditions*: 20.3:78.7:1.0 (v/v) methanol:water:acetic acid as mobile phase; flow rate, 1.0 mL/min; injection volume, 10 μL; detection wavelength, 280 nm; room temperature.

and 25 min. After the column was re-equilibrated with mobile phase, 30, 100 and 200 μL of the same model sample solution as above was respectively injected into the column and the column was eluted with 2% acetic acidic acetonitrile solution. The fraction with retention times from 10 to 25 min was collected and evaporated to dryness by rotary evaporator for purity analysis of chlorogenic acid. The result of HPLC analysis shown in Fig. 7 indicates that when injection volume of the model sample

Table 3
The relationship between the content of chlorogenic acid in the fraction and the injection volume

Injection volume (μL)	Contents of substrates in fraction (%)[a]				
	CGA	VA	PCA	GA	CA
50	98.7 ± 4.1	0.32 ± 0.09	0.11 ± 0.03	0.11 ± 0.04	0.26 ± 0.07
100	97.3 ± 4.4	0.46 ± 0.13	0.32 ± 0.07	0.25 ± 0.06	0.31 ± 0.11
200	96.0 ± 3.7	0.82 ± 0.22	0.41 ± 0.15	0.30 ± 0.14	0.42 ± 0.05
Extract[b]	85.6 ± 3.9	2.1 ± 0.52	1.4 ± 0.35	1.3 ± 0.43	4.5 ± 0.62

[a] All of collected fraction were dried by rotatory evaporation and the measurement of contents was carried out by re-dissolving the sample into methanol and determining by HPLC. CA, caffeic acid; GA, gallic acid; PCA, protocatechuic acid; VA, vanillic acid; CGA, chlorogenic acid.
[b] The contents of substrates investigated in the *Eucommia ulmodies* leaves extract.

solution is less than 50 μL, chlorogenic acid separated from the model mixture has a high purity.

On-line separation of chlorogenic acid from the *E. ulmodies* leaves extract by the molecularly imprinted polymer monolithic stationary phase using 2% acetic acidic acetonitrile as eluent is shown in Fig. 8. The fraction with retention times from 10 to 25 min was collected and evaporated to dryness as above. The purity of chlorogenic acid in the collected fraction was determined by high performance liquid chromatography with C_{18} column. Under different injection volumes, the contents of chlorogenic acid and the other co-existed compounds in the fractions collected are listed in Table 3. These results show that the contents of chlorogenic acid in the product processed by the molecularly imprinted polymer solid-phase adsorption are evidently higher than in the *E. ulmodies* leaves extract. Fig. 9a–c give the chromatograms of the standards, the extract and the dried fraction, respectively. Chlorogenic acid was eluted out at 20.3 min. The peaks with retention times of 5.6, 9.8, 22.6 and 28.9 min represent gallic acid (GA), protocatechuic acid (PCA), vanillic acid (VA) and caffeic acid (CA), respectively. The content of chlorogenic acid is much higher in dried fraction than in the *E. ulmodies* leaves extract. It shows that the separation and purification of chlorogenic acid from *E. ulmodies* leaves extract is successful by the molecularly imprinted polymer monolith.

4. Conclusion

The molecularly imprinted polymer monolithic stationary phase, prepared by an in situ method in the chromatographic column using caffeic acid as template, was successfully applied to the separation and purification of chlorogenic acid from *E. ulmodies* leaves extract by absorbing the impurities co-existed in the extract. The retention behavior of chlorogenic acid, the template and the impurities compounds co-existed in the leaves extract on the polymer monolith has been studied. The results show a weak adsorption of chlorogenic acid on the polymer monolith, which may result from a great "shape" difference of the chlorogenic acid molecule with the template molecule. This approach offered us a new way to separate and purify bioactive constituents from Traditional Chinese Medicine.

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